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The role of DMQ₉ in the long-lived mutant *clk-1*

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Abstract

Introduction—Ubiquinone (UQ) is a redox active lipid that transfers electrons from complex I or II to complex III in the electron transport chain (ETC). The long-lived *C. elegans* mutant *clk-1* is unable to synthesize its native ubiquinone, and accumulates high amounts of its precursor, 5-demethoxyubiquinone-9 (DMQ₉). In *clk-1*, complex I-III activity is inhibited while complex II-III activity is normal. We asked whether the complex I-III defect in *clk-1* was caused by: 1) a defect in the ETC; 2) an inhibitory effect of DMQ₉; or 3) a decreased amount of ubiquinone.

Methods—We extracted the endogenous quinones from wildtype (N2) and *clk-1* mitochondria, replenished them with exogenous ubiquinones, and measured ETC activities.

Results—Replenishment of extracted mutant and wildtype mitochondria resulted in equal enzymatic activities for complex I-III and II-III ETC assays. Blue native gels showed that supercomplex formation was indistinguishable between *clk-1* and N2. Addition of a pentane extract from *clk-1* mitochondria containing DMQ₉ to wildtype mitochondria specifically inhibited complex I-III activity. UQ in *clk-1* mitochondria was oxidized compared to N2.

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Author Contributions. YY performed the replenishment, ETC and oxidative phosphorylation assays and helped write the manuscript. VV performed the redox status assays for ubiquinone. JG performed the assays of ubiquinone levels in the mitochondria. SH developed the redox status assays, designed and interpreted the results of those experiments. EO performed additional ETC and oxidative phosphorylation experiments requested by reviewers. PGM and MMS were involved in the design and interpretation of all aspects of the experiments and wrote the manuscript. All authors were involved in editing the manuscript.

Discussion—Our results show that no measurable intrinsic ETC defect exists in *clk-1* mitochondria. The data indicate that DMQ₉ specifically inhibits electron transfer from complex I to ubiquinone.

Keywords

Mitochondria; longevity gene; *C. elegans*; aging; genetics; respiratory chain

1. Introduction

1.1 *clk-1*

The longevity of *C. elegans clk-1* mutant animals remains an enigma, despite intensive efforts to understand its underlying causes. *clk-1* was identified by Hekimi (Lakowski & Hekimi 1996; Ewbank *et al.* 1997) as the penultimate gene in the pathway synthesizing ubiquinone, a redox active lipid distributed in the membranes of eukaryotic cells (Crane 2007). One crucial function of UQ is to serve as a mobile electron carrier from complex I or II to complex III in mitochondria (Tran & Clarke 2007) (Supplemental Figure 1). Besides being an essential component of aerobic cellular respiration and ATP generation, UQ plays important roles in different biological processes. UQ acts as an antioxidant in its reduced form and eliminates lipid peroxyl radicals. Incompletely reduced UQ (semiubiquinone) acts as a pro-oxidant and can generate superoxide radicals (Genova *et al.* 2003; Sohal & Forster 2007). Deficiencies in CoQ cause a variety of clinical presentations, notably encephalomyopathy, cerebellar ataxia, myopathy, heart failure and infantile multisystemic disease (Quinzii *et al.* 2008; Quinzii & Hirano 2010; Fragaki *et al.* 2011). CoQ deficiency can also cause a mitochondrial deficiency syndrome through mitochondrial degradation (Rodriguez-Hernandez *et al.* 2009). Based on its crucial role in respiration and as an antioxidant, UQ is widely used as a therapeutic agent in treating mitochondria diseases (Marriage *et al.* 2004; Glover *et al.* 2010; Santa 2010), neurodegenerative diseases (Beal 2004; Earls *et al.* 2010) and cardiovascular diseases (Rosenfeldt *et al.* 2005; Yen *et al.* 2005; Sander *et al.* 2006; Molyneux *et al.* 2009).

Disruption of *clk-1* or its homolog in mice (*coq7*) eliminates UQ synthesis and causes accumulation of the intermediate in UQ₉ biosynthesis, 5-demethoxyubiquinone-9 (DMQ₉) (Miyadera *et al.* 2001). *clk-1* mutants in yeast (Padilla *et al.* 2004), *C. elegans* (Jonassen *et al.* 2001; Jonassen *et al.* 2002) and mice (Levavasseur *et al.* 2001) need UQ for normal development and survival. In nematodes, *clk-1* animals are dependent on ingestion of bacteria producing UQ₈ to replace the UQ₉ normally synthesized by the worm (Jonassen *et al.* 2001); feeding *clk-1* mutants UQ-less bacteria (GD1) results in larval lethality. Interestingly, loss of the enzyme COQ-3, a defect in UQ₉ synthesis upstream of *clk-1*, is lethal, indicating that both exogenous UQ and endogenous DMQ₉ are apparently necessary for survival of nematodes in the absence of UQ₉ synthesis (Hihi *et al.* 2002). However, studies on the role of DMQ in respiration are conflicting. Several studies have concluded that DMQ cannot substitute for UQ in respiration and redox function in mitochondrial (Jonassen *et al.* 2001; Jonassen *et al.* 2002; Padilla *et al.* 2004) or non-mitochondrial sites (Hihi *et al.* 2002; Arroyo *et al.* 2006). Others have detected some activity of complex I-III (but not II-III) in the presence of DMQ_X (Wallace and Young 1977; Levavasseur *et al.* 2001; Miyadera *et al.* 2001; Miyadera *et al.* 2002). Investigations of other functions of DMQ showed that DMQ₉ was unable to serve as an effective antioxidant for H₂O₂, though its ability to protect mitochondria from superoxide formation was not established. (Padilla *et al.* 2004)

1.2 Mitochondrial Function

We previously showed that mitochondrial complex I-dependent oxidative phosphorylation is inhibited in mitochondria from *clk-1* nematodes, while succinate driven respiration is unchanged relative to wild type. In addition, the electron transport activity of CI-III is severely depressed while that of CII-III remains normal (Kayser *et al.* 2004b; Yang *et al.* 2009). Given the role of UQ in transporting electrons from both complexes I and II to complex III, this was a surprising finding. Moreover, *clk-1* mitochondria containing UQ₉, display similar decreases in complex I dependent respiration as those containing UQ₈ (Yang *et al.* 2009). These mitochondria were obtained by feeding the *clk-1* animals bacteria engineered to produce UQ₉ (instead of UQ₈). Therefore the decrease in I-III activity is not the result of the specific change from UQ₉ to UQ₈ as an electron transporter in *clk-1* mutants. Whatever the cause of the complex I dependent defect, the result is an increase in ROS release in *clk-1* animals (Yang *et al.* 2009; Lee *et al.*, 2010). Recently, such an increase in ROS release has been implicated in extension of lifespan in *C. elegans* (Yang and Hekimi, 2010; Lee *et al.*, 2010).

1.3 Quinones

Dietary supplementation of *clk-1* animals with different UQs yielded relatively low amounts of exogenous UQs in *clk-1* mitochondria (Yang *et al.* 2009); the overwhelmingly predominant UQ species in *clk-1* mitochondria is, of course, DMQ₉. Previous studies showed that complex I had a higher K_m for ubiquinone than does complex II (Estornell *et al.* 1992; Lenaz 1998). As a result, the limiting amounts of exogenously supplied UQ available for electron transport in the *clk-1* animal possibly affect I-III activity more than II-III activity. It also remains possible that DMQ₉ competes with UQ specifically at complex I, but is less effective than UQ in electron transfer. This would result in DMQ₉ being a functional inhibitor of electron transport from CI-III, but not from CII-III.

In addition, Nakai *et al.* reported that a *coq7/clk-1* mutation in yeast led to disruption of mitochondrial structural integrity (Nakai *et al.* 2001). Loss of normal UQ₉ in *clk-1* mutants may interfere with formation or maintenance of intrinsic structures of the ETC including supercomplexes, which contain virtually all of the complex I found in *C. elegans* (Suthammarak *et al.* 2009). This distribution of complex I within supercomplexes may also limit the accessibility of any exogenously supplied quinones, *i.e.* quinones from the animal's diet, to complex I. In turn, this difference in accessibility could give rise to the measured difference in K_ms between complexes I and II for UQ.

We hypothesized that, if DMQ₉ inhibited I-III activity, removing DMQ₉ would eliminate the difference between N2 and *clk-1* mitochondria. We studied the possible effects of DMQ₉ by modifying a classic technique first described by Norling *et al.* (Norling *et al.* 1974). We extracted the endogenous quinones from wildtype and *clk-1* mitochondria with pentane. UQ₉ or UQ₁₀ was added back to reconstitute enzymatically active mitochondria. If the DMQ₉ is blocking electron transport in *clk-1* mitochondria, or if the amount of UQ is critical for electron transport from complex I to complex III, then the restored electron transport activity in UQ-replenished *clk-1* mitochondria will be the same as that in UQ-replenished N2 mitochondria. This finding then would also rule out that the ETC from *clk-1* mitochondria is intrinsically defective, since replenishment of extracted mitochondria with UQ should not eliminate the difference between N2 and *clk-1* I-III activity. We also studied the structure of supercomplexes in *clk-1* mitochondria via blue native gels to interrogate native mitochondria for intrinsic structural differences.

To differentiate between inhibitory effects of DMQ₉ and the effects of low ubiquinone concentrations we replenished N2 mitochondria with DMQ₉ extracted from *clk-1*

mitochondria. Finally, we measured the redox status of UQ in N2 and *clk-1* animals to determine if DMQ₉ inhibits transfer of electrons from complex I to ubiquinone, or from ubiquinone to complex III. Our data indicate that DMQ₉ specifically inhibits transfer of electrons from complex I to complex III.

2. Methods

2.1 Nematode strains

N2 (Bristol) and *clk-1(qm30)* were obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN, USA). *clk-1(qm30)* is the canonical null allele. Worms were grown on nematode growth media (NGM) agar with a lawn of *E. coli* as the food source, and maintained at 20°C. The engineered *E. coli* strains (Okada *et al.* 1997) used in this study were the kind gift of M. Kawamukai, Shimane University, Japan.

2.2 Pentane extraction

The experimental procedures were a modification of that published by Norling *et al.* (Norling *et al.* 1974). Mitochondria were first suspended in KME (100 mM KCl, 50 mM Mops, and 0.5 mM EGTA, pH 7.4) at 30 mg protein/mL and lyophilized for 8 hours. The lyophilized particles were suspended in cold pentane for 3 minutes with gentle homogenization. The pentane extract was removed by centrifugation (5100g, 5 minutes). To reconstitute UQ-depleted mitochondrial particles, the pellet was suspended with UQ₉ or UQ₁₀ (Sigma) in pentane at 40 nmol/g protein, shaken and incubated on ice for 5 minutes. The residual pentane and UQ were removed by centrifugation. UQ-depleted and UQ-replenished mitochondrial particles were dried under nitrogen and lyophilized for another hour to completely remove the pentane. UQ-depleted and UQ-replenished mitochondrial particles were rehydrated with KME for measurements of enzymatic activities. A portion of lyophilized mitochondria was rehydrated with KME, with no pentane extraction, as a control for maximal mitochondrial activity (L-mito in Figures).

DMQ₉ used in replenishment of UQ-depleted mitochondria (E-N2) was pentane extracted from *clk-1* mitochondria. 5mL of ice-cold pentane was added to 4mg of lyophilized *clk-1* mitochondria. After a gentle homogenization on ice for 5 minutes, the pentane extract was separated from mitochondria particles by centrifugation (5125g, 5 minutes). The extract was dried under nitrogen gas and resuspended in 500 µL cold pentane. 40 pmole of UQ₉ was added to the resuspended *clk-1* extract. The mix was added to 1mg of UQ depleted-N2 mitochondria. The procedures for the remainder of the replenishment were as described above.

Normally worms are fed either *E. coli* K12 (growth in liquid culture) or *E. coli* OP50 (growth on agar plates), each of which produces UQ₈. *clk-1* mitochondria that were replenished with UQ₉ were isolated from *clk-1* grown on engineered bacteria producing UQ₉ for at least two generations. Similarly, *clk-1* mitochondria that were replenished with UQ₁₀ were isolated from *clk-1* grown on engineered bacteria producing UQ₁₀. This was done in order to keep the primary ubiquinone species in the *clk-1* mitochondria the same before extraction and after replenishment. Since N2 makes UQ₉, and its complex I and II function is not altered by growth on wild type K12, or engineered UQ₁₀-producing bacteria, or its UQ₈ parental strain (Supplemental Figure 2), we fed N2 with K12 for these studies. For studies of the redox state of UQ in worms, N2 and *clk-1* were grown on UQ₉ engineered bacteria to minimize amounts of dietary UQ₈.

2.3 UQ quantification and identification

The UQ species in lyophilized, UQ-depleted, and UQ-replenished N2 or *clk-1* mitochondria were identified and quantified as described in Yang *et al.* (Yang *et al.* 2009). Samples were prepared by adding a known fixed amount of the internal standard di-propoxy-UQ₁₀ (diPrQ₁₀, a generous gift from Iain P. Hargreaves, Institute of Child Heath, London, United Kingdom) and 1-propanol (0.5mL for 400µg mitochondrial protein). 20µL of 2mg/mL *p*-benzoquinone were added to ensure that all the quinones were in the oxidized form. All the quinones (ubiquinones (UQ), demethoxyquinones (DMQ) and rhodoquinones (RQ)) were ionized by Atmospheric Pressure Chemical Ionization (APCI) and analyzed using a triple quadrupole mass spectrometer. Specific multiple reaction monitoring mode (MRM) transitions were performed, corresponding to *m/z* 864→197 (UQ₁₀), 920→253 (diPrQ₁₀), 795.5→197 (UQ₉), 765.5→166.9 (DMQ₉), 780.9→181.9 (RQ₉), 727.5→197 (UQ₈), 659.5→197 (UQ₇) and 591.5→197 (UQ₆), respectively. The samples were delivered to the API 4000 tandem mass spec (Applied Biosystems, Foster City, California, USA) using an Agilent 1200 series HPLC binary pump (Agilent technologies, Waldbronn, Germany) delivering 50% methanol:isopropanol at 0.5mL/min in flow injection analysis (FIA) mode. Samples were quantified against a 6 concentration levels calibration curve made with known amounts of UQ₆, UQ₉ and UQ₁₀ and the internal standard (diPrQ₁₀) spanning the concentration range found in the samples. UQ₇ was quantified assuming the same response in the detector as UQ₆. UQ₈, DMQ₉ and RQ₉ were quantified assuming the same response in the detector as UQ₉.

Reduced (UQ₉H₂) and oxidized UQ₉ ratios were assayed essentially as in (Ruiz-Jimenez *et al.* 2007) using a di-propoxy-CoQ₁₀ internal standard prepared as in (Duncan *et al.* 2005). The fresh frozen worm pellets were extracted in 100% methanol, incubated in ice for 15 min and spun for 5 min at 13,000 g in a microfuge at room temperature. The protein content of the pellets was assayed with Coomassie Plus (Pierce, IL, USA). The supernatant was assayed for oxidized and reduced UQ₉ levels after the addition of 120 ng/mL di-propoxy-UQ₁₀ internal standard. A 5 µL aliquot of each sample was injected at 7°C in a Waters Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 µm particles) maintained at 45°C using a Waters Acquity UPLC system. Analytes were eluted at 0.8 mL/min flow rate with an isocratic separation using 2 mM ammonium formate in 100% methanol for a total cycle time of 3.5 minutes. *clk-1* samples were assayed first and the reduced percentage was constant over the time of the assay. The effluent from the LC column was directly coupled to the electrospray source of a Waters Quattro Premier triple-quadrupole mass spectrometer running in positive ion mode. Identification and quantification of the analytes were carried out by monitoring the transition from the precursor to the product ions (multiple reaction monitoring mode, MRM). In particular the transitions of the precursor ions ammoniated adducts 812.6 *m/z* for UQ₉ and 814.6 *m/z* for UQ₉H₂ to the product ion 197 *m/z* for both analytes (Ruiz-Jimenez *et al.* 2007) were monitored in parallel with the transition of the di-propoxy-UQ₁₀ precursor ion 936.9 *m/z* to the product ion 253.2 *m/z*. Sample values of oxidized and reduced UQ₉ were normalized to mg of protein.

2.4 Mitochondrial electron transport chain (ETC) assays

The electron transport activities of UQ-depleted and UQ-replenished mitochondrial particles were measured by ETC assays as described in Hoppel *et al.* (Hoppel *et al.* 1987). Complex I and I-III activities use NADH as an electron donor; complex II and II-III activities use succinate as an electron donor. Lyophilization and pentane extraction followed by UQ replenishment irreversibly inactivates a portion of the mitochondrial proteins. In order to arrive at meaningful numbers for electron transport in replenished mitochondria, we normalized ubiquinone dependent complex I-III activity to the ubiquinone independent NFR (NADH-ferricyanide reductase) activity. The ubiquinone dependent complex II-III activity

was normalized to ubiquinone independent complex II activity. We then compared the normalized values for *clk-1* mitochondria to those for N2 mitochondria treated in a similar fashion.

2.5 Blue Native Gels

Mitochondrial electron transport chain structures of N2 and *clk-1* were analyzed by blue native gels following digitonin extraction as described previously. (Suthammarak *et al.* 2009; Suthammarak *et al.* 2010)

2.6 Statistical analysis

All data are presented as the mean of *n* experiments $\pm$ SD, with *n* $\geq$ 3. Data sets were individually compared using a student's t-test. Two sets of data were considered as statistically different when *p* $\leq$ 0.05.

3. Results

3.1 UQ replenished *clk-1* mitochondria have similar activities as UQ replenished N2 mitochondria

Before extraction, N2 mitochondria contained UQ₉ (Figure 1A) at >2,000 nmole/gm of mitochondrial protein. *clk-1* mitochondria contained DMQ₉, and low amounts of UQ₉ (Figure 1B,C). Following extraction, there were virtually no quinones in either *clk-1* or N2 mitochondria, indicating that pentane effectively removed the endogenous quinones (E-N2 and E-*clk-1* Figure 1A,B,C). Notably, essentially all DMQ₉ was removed from the *clk-1* mitochondria. After replenishment with specific UQs, both N2 and *clk-1* mitochondria possessed equivalent amounts of UQ₉ or UQ₁₀ in concentrations greater than in N2 before extraction (Figure 1A, B, C).

Simple lyophilization and rehydration of mitochondria from either N2 or *clk-1* animals results in absolute I-III and II-III enzyme activities that are virtually identical to their activities prior to any treatment (Supplemental Table). We used this lyophilized and rehydrated activity (shown as L-mito in Fig. 2A and 2B) as our baseline activity since all extracted samples were subjected to these treatments. Since pentane extraction decreases the amounts of complex I and complex II present (as measured by NFR and complex II activities, respectively) we normalized all post extraction complex I-III results to NFR (the amount of remaining complex I) and complex II-III results to complex II activity (the amount of remaining complex II) in Figure 2. Pentane extraction unaccompanied by quinone replenishment nearly abolished complex I-III and complex II-III activities (Supplemental Figure 3). After extraction and replenishment with UQ₉, the normalized activities (see above and Methods) of reconstituted *clk-1* and N2 mitochondria were similar to each other (Figure 2A, +UQ₉). This represented 130% of the activity of *clk-1* mitochondria prior to extraction (Figure 2C), but only 30% of N2's complex I-III activity prior to extraction. To determine whether I-III or II-III activities are dependent on the UQ species after this treatment, we also reconstituted N2 and *clk-1* mitochondria with UQ₁₀. I-III activity in N2 was higher than in *clk-1* UQ₁₀-replenished mitochondria (Figure 2A, +UQ₁₀). This result suggests that either DMQ₉ interferes with electron transport from complex I to III, or that the decreased amounts of ubiquinone ordinarily present in *clk-1* mitochondria are limiting for complex I-III activity.

Complex II-III activities were restored to normal levels in both N2 mitochondria and *clk-1* mitochondria after pentane extraction and replenishment with UQ₉ or UQ₁₀ (Figure 2B, +UQ₉, 2D, +UQ₁₀). No relative improvement was seen in complex II-III activity in *clk-1* mitochondria compared to N2 mitochondria with UQ₉. Complex II-III activity of *clk-1* mitochondria was greater when replenished with UQ₁₀ compared with UQ₉ (Figure 2D,

+UQ10). This rate increase is similar to the increase in I-III activity with both UQ₉ and UQ₁₀. There was no increase in II-III activity when CoQ9 was used for the replenishment compared to the activity before pentane extraction. The cause of this difference is unclear, though it may indicate that UQ₁₀ is a better substrate for nematode complex II than is UQ₉.

3.2 Blue Native Gels

Blue native gel analysis showed no differences between N2 and *clk-1* mitochondria (Figure 3). In particular, the high molecular weight bands containing supercomplexes I:III:IV were identical between the two strains. Previous studies have shown that defects in complexes I, III, or IV can result in significant alterations in banding patterns of blue native gels (Falk *et al.* 2009; Suthammarak *et al.* 2009; Suthammarak *et al.* 2010).

3.3 DMQ₉ inhibits electron transport from complex I to UQ

To investigate the effects of DMQ₉ on electron transport, we added pentane extracts from *clk-1* mitochondria (containing DMQ₉) to UQ₉. This mixture was used to replenish pentane extracted N2 mitochondria. The amounts of UQ₉ in mitochondria replenished with either UQ₉ alone or UQ₉ plus DMQ₉ were similar (Figure 4). However, the amount of DMQ₉ in the replenished mitochondria was less than that normally found in *clk-1* mitochondria (*cf.* Figure 1B with Figure 4). After normalizing complex I-III activity to NFR, a measure of complex I enzymatic activity in the preparation, the average I-III activity from mitochondria replenished with UQ₉ and DMQ₉ was decreased compared to UQ₉-replenished N2 mitochondria (Figure 5A). Addition of a pentane extract from N2 mitochondria to UQ₉-replenished N2 mitochondria did not decrease activity (Supplemental Figure 3). This result indicates that an extract from *clk-1* mitochondria specifically inhibits complex I-III activity while an extract containing essentially UQ₉ as its only quinone species does not. In contrast, the normalized complex II-III activity of UQ₉-replenished mitochondria with or without DMQ₉ are 1.9 and 1.6, respectively (Figure 5B), indicating that complex II-III activity was not significantly affected by the addition of DMQ₉.

3.4 The percentage of reduced UQ₉ is less in *clk-1* than in N2

Previous work from whole worm extraction of UQ showed that a defect in complex I function in *C. elegans* led to a decrease in the reduced form of UQ, whereas a defect in complex III led to an increase in the amount of reduced UQ (Vasta *et al.* 2011). Thus, while preparations of UQ from whole worms include UQ from both inside and outside the mitochondria, clearly the assay can detect changes in UQ within mitochondria. We reasoned that if DMQ₉ inhibits electron transfer from complex I to UQ, the percentage of reduced UQ in *clk-1* animals will be decreased compared with that in N2. The percentage of reduced UQ in *clk-1* and N2 worms, both fed with engineered bacteria producing UQ₉, was measured in whole worms (Figure 6). The reduced UQ₉ in N2 is 74.6 ± 9.1 % of the total UQ₉. The reduced UQ₉ in *clk-1* is 57.7 ± 8.7 % of the total UQ₉ ($p = 0.05$). The decrease in the percentage of reduced UQ in *clk-1* suggests that an inhibitor of electron transport from complex I to UQ exists in *clk-1*.

4. Discussion

In this study, we sought to understand how complex I-III activity is deficient in *clk-1* animals, while complex II-III activity is normal. There are several possible mechanisms for this phenomenon: 1) *clk-1* mitochondria are intrinsically altered such that complex I-III activity is defective. 2) DMQ₉ specifically inhibits the transfer of electrons from complex I to UQ or from UQ to complex III. 3) The levels of exogenous UQ exchanging with endogenous quinones within the supercomplex I:III:IV in *clk-1* mitochondria are too low to support efficient electron transport. Transfer from complex II-III is less affected either

because complex II has a lower K_m for quinones (Estornell *et al.* 1992), or because the exogenous (*i.e.* bacterial) quinones can reach complex II more readily than complex I. In effect, a separate UQ pool may be available for complex I compared to complex II for exogenously supplied quinones (Estornell *et al.* 1992; Lenaz 1998). Any differences in exogenous UQ availability for transfer of electrons from complexes I versus II may in fact underlie the differences in ubiquinone related K_m s between the complexes. Below we discuss each of these possibilities separately.

4.1 Intrinsic changes in *clk-1* mitochondria

After pentane extraction of quinones from mitochondria, addition of UQ₉ (or UQ₁₀) restored complex I-III activity to similar levels in N2 and *clk-1* mitochondria. This indicated that no initial differences existed between the ETC components *per se* that accounted for the difference in complex I-III activities between N2 and *clk-1* mitochondria. However, we also sought corroboration from native preparations of mitochondria. Previous work suggested that *clk-1* may actually affect mitochondrial structure (Nakai *et al.* 2001). We and others had already established that complex I activity may be significantly reduced when supercomplex I:III:IV formation is defective (Schafer *et al.* 2006; Suthammarak *et al.* 2009). Although isolated complex I activity is normal in *clk-1* mitochondria (Kayser *et al.* 2004b), we wondered whether changes in supercomplex formation could explain the I-III defect. We found no apparent differences between N2 and *clk-1* animals in supercomplex formation, in contrast to studies of the effects of defects in complexes I, III and IV (Falk *et al.* 2008; Suthammarak *et al.* 2009; Suthammarak *et al.* 2010).

4.2 Inhibition by DMQ₉

The similar I-III activity in N2 and *clk-1* mitochondria following removal of DMQ₉ and replenishment with UQ is clearly consistent with an inhibitory effect of DMQ₉ on the ETC. However, decreased amounts of functional ubiquinone in *clk-1* mitochondria may also produce this effect. Ideally, kinetic studies with varying amounts of UQ to determine V_{max} and K_m values for UQ could resolve this dilemma. However, we are unable to titrate the amount of UQ in the I-III supercomplex to perform these experiments. Consequently, we can not assign DMQ as a classical competitive inhibitor for UQ. As an alternative approach to determine whether DMQ₉ inhibits electron transport from complex I to III, it would be helpful to measure electron transport activity in reconstituted mitochondria that have equal amounts of DMQ₉ and UQ₉ present. Due to the unavailability of HPLC-purified DMQ₉, this approach is also not technically feasible. Thus, we extracted the quinone species from *clk-1* mitochondria (containing DMQ₉) and replenished N2 mitochondria with the extract. Compared to N2 mitochondria replenished with UQ₉ alone or with an N2 extract, we found that the addition of DMQ₉ produced a lower complex I-III activity. Our data lend support to the studies that have previously found that DMQ does not substitute for UQ in respiration and redox function in the mitochondrial ETC (Jonassen *et al.* 2001; Padilla *et al.* 2004; Jonassen *et al.* 2002). It is possible that DMQ₉ has limited electron transport activity and appears to inhibit UQ by competition for identical sites on complex I. It is also possible that an unidentified extracted compound in *clk-1* mitochondria specifically inhibits complex I-III activity. However, this molecule must be specific to *clk-1*, since an N2 mitochondrial extract did not inhibit function.

DMQ₉ could inhibit complex I-III activity either by blocking transfer of electrons from complex I to UQ₉, or by interfering with the ability of UQ₉ to pass electrons to complex III. To determine the location of the inhibition, we measured the percentage of reduced UQ in *clk-1* animals. Vasta *et al.* previously identified the ratio of reduced and oxidized UQ in different *C. elegans* mitochondrial mutants (Vasta *et al.* 2011). In N2 grown on K12 bacteria UQ₉ was 84% reduced. In *isp-1*, a mutant severely deficient in complex III function, 90% of

UQ was in the reduced form. *isp-1* contains a primary mutation in the Rieske iron-sulfur protein subunit of complex III (Feng *et al.* 2001). Furthermore, the complex I mutant *gas-1* showed only 45% of total UQ in the reduced form; the complex II mutant *mev-1* had 55% reduced UQ. Thus, the percentage of reduced UQ correlates with the location of the defect in the electron transport chain.

If DMQ₉ interferes with the electron transport from complex I to UQ, a decrease in the percentage of UQ in the reduced form should be observed in *clk-1* animals when compared with N2. Consistent with this model, the percentage of reduced UQ in *clk-1* is lower than that in N2 animals. Of course, we are measuring UQ from extra-mitochondrial sources as well as from mitochondria. However, we have previously shown that with this technique we can unequivocally measure CoQ redox states that are consistent with the location of specific ETC defects (Vasta *et al.* 2011). Therefore, we think that a comparison of *clk-1*'s redox status to measurements in N2 gives accurate information about the location of *clk-1*'s ETC defect. In addition, DMQ₉ may inhibit UQ reductases other than complex I or complex II. These potential targets include glutathione reductase, thioredoxin reductase and others (Turunen *et al.* 2004). However, given that other ETC defects *gas-1* and *mev-1* predictably decrease the percentage of reduced UQ₉, we think that the most reasonable target for DMQ₉ inhibition of I-III activity remains mitochondrial complex I. We conclude that DMQ₉ inhibits the transfer of electrons from complex I to UQ₉, as opposed to their subsequent transfer to complex III. Therefore, the defective complex I-III activity that has been observed in *clk-1* mitochondria results at least in part from complex I-specific inhibitory effects of DMQ₉.

4.3 Low levels of UQ

A limitation of our study is that we have not completely ruled out that the low levels of UQ in *clk-1* mitochondria may contribute to the specific decrease in I-III activity compared to II-III activity. We interpret the redox status of UQ in *clk-1* mitochondria to indicate that this possibility is less likely than inhibition of I-III function by DMQ₉. However, it remains possible that the majority of dietary quinone in *clk-1* mitochondria, or even replenished quinone, is excluded from the I:III:IV supercomplex, which is the predominant form of complex I in *C. elegans* (Suthammarak *et al.* 2009). Complex II, in nematodes, is not found as part of a supercomplex (Suthammarak *et al.* 2009), and therefore may be more accessible to exogenously supplied quinones. In addition, Lenaz earlier showed that the K_m for reducing ubiquinone is 10 fold less for complex II than for complex I (Estornell *et al.* 1992; Lenaz 1998). Thus, the low amounts of UQ in *clk-1* mitochondria are likely to affect complex I more than complex II. Since Lenaz determined the kinetics of quinone binding by adding quinones to mitochondria, the high K_m of complex I for ubiquinone may also result from limited access of ubiquinones to complex I in the supercomplex. The decreased I-III activity of *clk-1* mitochondria may be in part due to inhibition by DMQ₉ as well as low amounts of UQ₉ in the I:III:IV supercomplex. Dietary UQ may only be supporting complex II dependent respiration in *clk-1* mitochondria. This may account for the decreased free radical damage ordinarily seen in *clk-1* mitochondrial proteins (Yang *et al.* 2009), and perhaps underlies the basic CLK phenotypes.

Our results must be interpreted in light of the low total complex I-III activity regained in replenished mitochondria. This may represent either damage to the ETC by the pentane extraction or inadequate delivery of replenished quinone to the I:III:IV supercomplex. If damage, it appears also to be specific to complex I-III activity. Complex II, II-III, and NFR activities were very well maintained after replenishment, indicating that the mitochondria are not so extensively damaged as to eliminate other complex enzymatic functions. Regardless, damage should be the same in both N2 and *clk-1* mitochondria. Therefore,

comparison of these strains following UQ-replenishment is the most accurate measure of the effects of extracting DMQ₉ available to us.

These results superficially conflict with a report by Branicky *et al.* (Branicky *et al.* 2006). They found a group of tRNA suppressors that improved many of the phenotypes of *clk-1(e2519)*, including production of a normal number of offspring on Q-less bacteria. They concluded that the suppressed animals synthesized a small amount of UQ₉, which they measured in extracts from whole worms grown on Q₈-replete bacteria, OP50. They did not see any UQ₉ in unsuppressed *clk-1* animals grown on OP50. However, there are no data that show UQ₉ synthesis from *clk-1* animals grown on Q-less bacteria. We have measured similar amounts of UQ₉ in mitochondria isolated from ordinary cultures of the null allele of *clk-1(qm30)* grown on UQ₈-producing bacteria (Yang *et al.* 2009). In this case the UQ₉ we measure in mitochondria must represent an exogenous contribution from their diet. We have in fact measured UQ₉ in K12 and OP50 bacterial cultures (Yang *et al.* 2009). It is possible that the suppressors are working through effects other than restoring enzymatic activity of *clk-1* animals. Regardless, the suppression did not significantly decrease levels of DMQ₉ in whole worm extracts. They interpreted their results to indicate that DMQ₉ did not contribute to the *clk-1* phenotype. Nevertheless, our data are easily reconciled with that of Branicky *et al.*, and in fact the sum of the data may give a clear picture of the role of exogenous versus endogenously supplied quinones. If the suppressed animals are in fact restoring *clk-1* enzymatic activity, it may be that a small amount of *endogenously* synthesized UQ₉ is sufficient to repair the *Clk-1* phenotype, while exogenously supplied ubiquinone is not. We hypothesize that endogenous UQ₉ can reach the I:III:IV supercomplex, while dietary UQ₉ cannot. If this is the case, then endogenous UQ₉ may out compete DMQ₉ and allow I-III activity to be specifically rescued.

Our data lead us to conclude that a specific block of complex I activity is due to an inability of exogenous UQ from the diet to reach the I:III:IV supercomplex because limiting amounts of UQ cannot displace endogenous DMQ₉, a poor electron carrier. If all endogenous quinones are extracted the *clk-1* ETC can function as well as wildtype. DMQ₉ specifically inhibits electron flow from complex I to complex III by blocking offloading of electrons from complex I to UQ.

4.4 Conclusions

Ultimately, studies of mitochondrial function in *clk-1* animals must address whether the data can explain the animal's longevity. Previously we reported that *clk-1* mitochondria generated more ROS but accumulated remarkably less oxidative damage to mitochondrial proteins (Yang *et al.* 2009). Moreover, the double mutant *clk-1; gas-1* is extremely long-lived (Kayser *et al.* 2004a). *gas-1* has complex I dependent rates of oxidative phosphorylation that are nearly identical to that of *clk-1*, but in contrast to *clk-1*, is very short lived and accumulates extensive oxidative damage (Kayser *et al.* 2001; Hartman *et al.* 2001). *Since clk-1* reversed the short-lived phenotype of *gas-1*, and strongly decreases accumulated HNE damage, there is very likely an effective antioxidant in *clk-1* worms. There are several possible candidates, though the *sod* genes seem to have been eliminated by the results of Yang (Yang *et al.* 2007). It is still possible that glutathione transferase, catalase, DMQ₉ or another scavenger serve this role in *clk-1*. Although ROS production and damage do not always correlate with lifespan (Yang *et al.* 2007; Doonan *et al.* 2008), in *clk-1* **decreased** electron transport through complex I is paired with **increased** ROS scavenging. This unique combination is the simplest explanation of the animal's longevity. The decrease in complex I activity appears to be due to an inhibitory effect of DMQ₉ on I-III activity.

Highlights

> Complex I-III activity is defective in *clk-1* mitochondria. > Removing DMQ₉ from *clk-1* mitochondria improves complex I activity. > Supercomplex structure is similar in N2 and *clk-1*. > DMQ₉ inhibits complex I-III activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

UQ	Ubiquinone
DMQ	Demethoxyubiquinone
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CI	complex I
CII	complex II
N2	wildtype <i>C. elegans</i>

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Figure 1. The amount of UQ in pentane extracted/replenished N2 and *clk-1* mitochondria
(A) The amount of UQ measured in: L-N2 = lyophilized and rehydrated N2 mitochondria; E-N2 = pentane extracted N2 mitochondria; E-N2+UQ₉ = pentane extracted N2 mitochondria reconstituted with UQ₉. The majority of UQ measured in N2 mitochondria before extraction and after extraction and replenishment is UQ₉. **(B)** The same experimental procedures were performed using mitochondria isolated from *clk-1* grown on UQ₉. The majority of UQ measured in *clk-1* mitochondria before extraction is the biosynthetic precursor of UQ₉, DMQ₉. L-*clk-1* = lyophilized and rehydrated mitochondria from *clk-1* grown on UQ₉. E-*clk-1* = pentane extracted mitochondria from *clk-1* grown on UQ₉. E-*clk-1*+UQ₉ = pentane extracted mitochondria from *clk-1* grown on UQ₉ replenished with

UQ₉(C) The same experimental procedures were performed using mitochondria isolated from *clk-1* grown on UQ₁₀. The majority of UQ measured in *clk-1* mitochondria before extraction is the biosynthetic precursor of UQ₉, DMQ₉. L-*clk-1* = lyophilized and rehydrated mitochondria from *clk-1* grown on UQ₁₀. E-*clk-1* = pentane extracted mitochondria from *clk-1* grown on UQ₁₀. E-*clk-1*+UQ₁₀ = pentane extracted mitochondria from *clk-1* grown on UQ₁₀ replenished with UQ₁₀. Data shown here are means $\pm$ standard deviation. Each sample was measured in duplicate from 3 – 5 independent pentane extraction/replenishment experiments. Numbers over largest peaks refer to the values (nmoles/g protein) of DMQ_X or UQ_X of the particular experiment and are added for clarification.

Figure 2. *clk-1* mitochondria showed the same electron transport activities as N2 mitochondria after removing DMQ₉.

After pentane extraction and replenishment with UQ₉, activities of (A) complex I-III and (B) complex II-III were measured in N2 and *clk-1* mitochondria. The activities were normalized to either NFR (for I-III) or CII (for II-III) activity. After pentane extraction and replenishment with UQ₉, the normalized I-III (A) and II-III (B) activities showed no difference between *clk-1* and N2 mitochondria. After replenishment with UQ₁₀, N2 mitochondria had significantly higher activity complex I-III than *clk-1* mitochondria. (C) Pentane extracted/replenished *clk-1* mitochondria, regardless of whether they were replenished with UQ₉ or UQ₁₀, showed a greater percentage of recovery in complex I-III

activity than did N2 when compared with lyophilized and rehydrated mitochondria (* indicates $p < 0.05$). **(D)** Pentane extracted *clk-1* and N2 mitochondria replenished with UQ₉ showed a similar percentage of recovery of complex II-III activity compared to lyophilized samples. *clk-1* mitochondria replenished with UQ₁₀ showed a greater proportional increase in II-III activity than did N2. Data shown here are means $\pm$ standard deviations. Each sample was measured in duplicate from 4 – 7 pentane extraction/replenishment experiments. L-mito= lyophilized mitochondria. E-mito = pentane-extracted mitochondria. +UQ₉ or +UQ₁₀ refers to the ubiquinone used for replenishment.

Figure 3. The organization of respiratory complexes in N2 and *clk-1*

Representative digitonin based blue native gel (from n=3 independent gels) of mitochondrial proteins from N2 and *clk-1* as described in Suthammarak *et.al.* (Suthammarak *et al.* 2009). The numbers in the right panel indicate the approximate molecular masses (kDa) of the corresponding protein bands. On the left are the identities of the bands as determined by mass spectrometry in N2. (Suthammarak *et al.* 2009). No differences were noted between N2 and *clk-1*.

Figure 4. The quinone profile in mitochondria replenished with UQ₉ and DMQ₉

DMQ₉ was extracted from *clk-1* mitochondria and added with UQ₉ to pentane-extracted N2 mitochondria. Mitochondria incorporated DMQ₉ along with UQ₉. L-N2 = lyophilized N2 mitochondria. E-N2 = pentane-extracted N2 mitochondria. E-N2+UQ₉ = pentane extracted N2 mitochondria reconstituted with UQ₉. E-N2+UQ₉+DMQ₉ = pentane extracted N2 mitochondria reconstituted with UQ₉ and DMQ₉ extracted from *clk-1* mitochondria.

Figure 5. Mitochondrial activities measured with or without DMQ₉

Complex I-III (A) and II-III (B) activities of UQ₉-replenished mitochondria were measured with or without DMQ₉ added to the replenishment mixture. The complex I-III and II-III activities shown here were normalized to NFR and CII activities, respectively. Complex I-III activity was decreased in the presence of DMQ₉ ($p = 0.05$). Complex II-III activity was indistinguishable between mitochondria with or without DMQ₉. Data shown here are mean $\pm$ standard deviation. Each sample was measured in duplicate from 3 – 5 pentane extraction/replenishment experiments. E-N2+UQ₉ = pentane extracted N2 mitochondria reconstituted with UQ₉. E-N2+UQ₉+DMQ₉ = pentane extracted N2 mitochondria reconstituted with UQ₉ and DMQ₉ extracted from *clk-1* mitochondria.

Figure 6. The percentage of reduced UQ₉ is decreased in *clk-1* compared with N2

The percentage of reduced UQ₉ was determined in *clk-1* and N2 worms. The percentage of reduced UQ₉ in the *clk-1* mutant is $57.7 \pm 8.7\%$ of the total UQ, which is lower than that in N2 ($74.6 \pm 9.1\%$, $p < 0.05$). Data shown here are means $\pm$ standard deviations from 4–6 different worm cultures of N2 and *clk-1*.